

1.5 ml of the overnight culture were collected by centrifugation for 2 min. in a microcentrifuge (14K rpm), the supernatant was discarded and the cell pellet was resuspended in 50 µl TE buffer with 10 µg/ml RNase A (Pharmacia). One hundred microliters of a solution containing 0.2 N NaOH, 1% SDS was added and the cells were lysed for 2 min. The lysate was gently mixed with 100 µl of 1.32 M potassium acetate, pH 4.8, and the mixture was centrifuged for 4 min. in a microcentrifuge (14K rpm); the pellet comprising cell debris was discarded. Plasmid DNA was precipitated from the supernatant with 200 µl ethanol and pelleted by centrifugation a microcentrifuge (14K rpm). The DNA pellet was air dried for 15 min. and was then redissolved in 50 µl TE buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA).

**b) Reamplification Of HCV Clones To Add The Phage
T7 Promoter For Subsequent *In Vitro* Transcription**

To ensure that the RNA product of transcription had a discrete 3' end it was necessary to create linear transcription templates which stopped at the end of the HCV sequence. These fragments were conveniently produced using the PCR to reamplify the segment of the plasmid containing the phage promoter sequence and the HCV insert. For these studies, the clone of HCV type Δ1c was reamplified using a primer that hybridizes to the T7 promoter sequence: 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO:55; "the T7 promoter primer") (Novagen) in combination with the 3' terminal HCV-specific primer HCV308 (SEQ ID NO:54). For these reactions, 1 µl of plasmid DNA (approximately 10 to 100 ng) was reamplified in a 200 µl PCR using the T7 and HCV308 primers as described above with the exception that 30 cycles of amplification were employed. The resulting amplicon was 354 bp in length. After amplification the PCR mixture was transferred to a fresh 1.5 ml microcentrifuge tube, the mixture was brought to a final concentration of 2 M NH₄OAc, and the products were precipitated by the addition of one volume of 100% isopropanol. Following a 10 min. incubation at room temperature, the precipitates were collected by centrifugation, washed once with 80% ethanol and dried under vacuum. The collected material was dissolved in 100 µl nuclease-free distilled water (Promega).

Segments of RNA were produced from this amplicon by *in vitro* transcription using the RiboMAX™ Large Scale RNA Production System (Promega) in accordance with the manufacturer's instructions, using 5.3 µg of the amplicon described above in a 100 µl reaction. The transcription reaction was incubated for 3.75 hours, after which the DNA template was destroyed by the addition of 5-6 µl of RQ1 RNase-free DNase (1 unit/µl) according to the RiboMAX™ kit instructions. The reaction was extracted twice with phenol/chloroform/isoamyl alcohol (50:48:2) and the aqueous phase was transferred to a fresh microcentrifuge tube. The RNA was then collected by the addition of 10 µl of 3M NH₄OAc, pH 5.2 and 110 µl of 100% isopropanol. Following a 5 min. incubation at 4°C, the precipitate was collected by centrifugation, washed once with 80% ethanol and dried under vacuum. The sequence of the resulting RNA transcript (HCV1.1 transcript) is listed in SEQ ID NO:56.

c) Detection Of The HCV1.1 Transcript In The Invader-Directed Cleavage Assay

Detection of the HCV1.1 transcript was tested in the invader-directed cleavage assay using an HCV-specific probe oligonucleotide [5'-CCGGTCGTCCTGGCAATXCC-3' (SEQ ID NO:57); X indicates the presence of a fluorescein dye on an abasic linker) and an HCV-specific invader oligonucleotide [5'-GTTTATCCAAGAAAGGACCCGGTCC-3' (SEQ ID NO:58)] that causes a 6-nucleotide invasive cleavage of the probe.

Each 10 µl of reaction mixture comprised 5 pmole of the probe oligonucleotide (SEQ ID NO:57) and 10 pmole of the invader oligonucleotide (SEQ ID NO:58) in a buffer of 10 mM MOPS, pH 7.5 with 50 mM KCl, 4 mM MnCl₂, 0.05% each Tween-20 and Nonidet-P40 and 7.8 units RNasin® ribonuclease inhibitor (Promega). The cleavage agents employed were Cleavase® A/G (used at 5.3 ng/10 µl reaction) or DNAPTth (used at 5 polymerase units/10 µl reaction). The amount of RNA target was varied as indicated below. When RNase treatment is indicated, the target RNAs were pre-treated with 10 µg of RNase A (Sigma) at 37°C for 30 min. to demonstrate that the detection was specific for the RNA in the reaction and not due to the presence

of any residual DNA template from the transcription reaction. RNase-treated aliquots of the HCV RNA were used directly without intervening purification.

For each reaction, the target RNAs were suspended in the reaction solutions as described above, but lacking the cleavage agent and the $MnCl_2$ for a final volume of 10 μ l, with the invader and probe at the concentrations listed above. The reactions were warmed to 46°C and the reactions were started by the addition of a mixture of the appropriate enzyme with $MnCl_2$. After incubation for 30 min. at 46°C, the reactions were stopped by the addition of 8 μ l of 95% formamide, 10 mM EDTA and 0.02% methyl violet (methyl violet loading buffer). Samples were then resolved by electrophoresis through a 15% denaturing polyacrylamide gel (19:1 cross-linked), containing 7 M urea, in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4 mM EDTA. Following electrophoresis, the labeled reaction products were visualized using the FMBIO-100 Image Analyzer (Hitachi), with the resulting imager scan shown in Figure 52.

In Figure 52, the samples analyzed in lanes 1-4 contained 1 pmole of the RNA target, the reactions shown in lanes 5-8 contained 100 fmoles of the RNA target and the reactions shown in lanes 9-12 contained 10 fmoles of the RNA target. All odd-numbered lanes depict reactions performed using Cleavase® A/G enzyme and all even-numbered lanes depict reactions performed using DNAPTth. The reactions analyzed in lanes 1, 2, 5, 6, 9 and 10 contained RNA that had been pre-digested with RNase A. These data demonstrate that the invasive cleavage reaction efficiently detects RNA targets and further, the absence of any specific cleavage signal in the RNase-treated samples confirms that the specific cleavage product seen in the other lanes is dependent upon the presence of input RNA.